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THE EFFECT OF THE NITROSOUREA ANALOG OF THYMIDINE, 3′-[3-(2-CHLOROETHYL)-3-NITROSOUREIDO]-3′-DEOXYTHYMIDINE, ON *ESCHERICHIA COLI* THYMIDINE KINASE

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Summary

Escherichia coli thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) is irreversibly inactivated by incubation with 3′-[3-(2-chloroethyl)-3-nitrosoureido]-3′-deoxythymidine (3′-CTNU). The inactivation of the enzyme followed first-order kinetics even after loss of 96% of the original activity. This indicates that the inactivation process is a one-kill phenomenon and not a generation of less active enzyme. The addition of a preincubated aqueous solution of 3′-CTNU to the enzyme reaction mixture did not inactivate the enzyme. ATP · Mg^{2+} but not thymidine protects the enzyme from inactivation by 3′-CTNU. The allosteric regulators, dTTP, dCTP and dCDP also afforded complete protection of the enzyme from inactivation by 3′-CTNU. These data indicate that the dimer form of the enzyme is completely resistant to inactivation by 3′-CTNU, but the monomer form of the enzyme is sensitive. The specificity of the protection is supported by the finding that neither ATP · Mg^{2+} nor thymidine protect yeast alcohol dehydrogenase from inactivation by this nitrosourea analog of thymidine.

Introduction

The chloroethylnitrosourea analogs are an important class of antitumor agent which include 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose (Streptozotocin), for example. Although the penultimate event responsible for the antineoplastic actions of nitrosoureas is not known, the effectiveness of nitrosoureas is presumably due to their abilities to

alkylate or carbamoylate a critical cellular component [1]. The synthesis and biological activities of a new class of nitrosoureas have been reported [2], in which the deoxyribonucleoside thymidine is used as the carrier of the nitrosourea functionality in either the 3'- and 5'-position of the deoxyribose moiety. Montgomery and Thomas [3] recently reported the synthesis of methylnitrosourea analogs of ribonucleosides. It was subsequently shown by Fischer et al. [4] that the cytotoxicity of the nitrosourea analogs of thymidine could be prevented by pyrimidine deoxyribonucleosides but not by pyrimidine ribonucleosides, purine ribonucleosides or purine deoxyribonucleosides. This report describes the interaction of 3'-[3-(2-chloroethyl)-3-nitrosoureido]-3'-deoxythymidine with the macromolecule, *E. coli* thymidine kinase, as a part of a program designed to understand the mechanism for its cytotoxicity as well as anticancer activity.

Materials and Methods

Materials. 3'-CTNU was synthesized by the procedure reported previously [2]. Thymidine kinase from *E. coli* cells was subjected to the six steps of purification (heat, streptomycin, first and second $(NH_4)_2SO_4$ fractionation, and DEAE-cellulose) described by Okazaki and Kornberg [5]. [2-^{14}C]Thymidine (specific activity; 50 mCi/mmol) was obtained from Moravek Biochemicals. Yeast alcohol dehydrogenase, dCDP, dCTP and CTP were purchased from Sigma Chemical Co., and CDP, dTTP, ATP and dTMP from P-L Biochemicals. All other chemicals were reagent grade and used without further purification.

Enzyme assay. The assay for thymidine kinase activity measured the conversion of [2-^{14}C]thymidine to [2-^{14}C]thymidylate. Thymidine kinase assay mixture contained 100 mM, Tris-HCl buffer, pH 7.5, 3 mM $MgCl_2$, 3 mM ATP, 50 μM [2-^{14}C]thymidine (50 μCi/μmol), 0.3 mg/ml bovine serum albumin; 2% glycerol; 1 mM β-mercaptoethanol and a preparation of enzyme in a total volume of 0.1 ml. dTMP, the product formed, was separated by two procedures: (i) by adsorption onto a DEAE-cellulose disc according to the method of Furlong [6]; (ii) by separation of thymidine and dTMP from each other by TLC on cellulose Polygram Cel 300 PEI/UV with 0.4 M LiCl as the developing solvent [7,8]. The area corresponding to dTMP was cut out and counted in a Beckman LS7500 liquid scintillation spectrometer. 1 unit of enzyme activity is equal to the formation of 1 nmol dTMP per min at 37°C.

Yeast alcohol dehydrogenase was assayed by spectroscopic measurement of the formation of NADH from NAD at 340 nm, at 30°C. The assay mixture contained 0.017 M pyrophosphate, pH 8.8/0.35 M ethanol/0.008 M NAD. 1 unit enzyme activity is equal to the formation of 1 μmol acetaldehyde per min at pH 8.8, at 25°C.

Results

The effect of 3'-CTNU, ATP · Mg^{2+} and thymidine on thymidine kinase activity. Thymidine kinase was inactivated by 3'-CTNU, but whereas ATP · Mg^{2+} afforded complete protection thymidine was inactive (Table I). The addition of a preincubated solution of 3'-CTNU to the enzyme reaction mixture did

TABLE I

EFFECT OF ATP · Mg^{2+} AND THYMIDINE ON THE INACTIVATION OF *E. COLI* THYMIDINE KINASE BY 3'-CTNU

E. coli thymidine kinase was incubated at 37°C for 15 min with the indicated compounds. 3'-CTNU was added to the incubation mixture which contained *E. coli* thymidine kinase (0.18 units), 100 mM Tris-HCl, pH 7.5, and 2.5 mg/ml bovine serum albumin. Thymidine kinase activities were determined as described in the text.

Concentration (mM)			Percent activity remaining
3'-CTNU	ATP · Mg^{2+}	Thymidine	
3.92	0	0	18
3.92	9	0	102
3.92	0	2.25	11
3.92	9	2.25	101
0	9	0	100
0	0	2.25	90
0	9	2.25	99
0	0	0	100

not inactivate the enzyme. The linearity of inactivation of the thymidine kinase activity by two levels of 3'-CTNU is depicted in Fig. 1A and supports first-order inactivation kinetics. The effect of the concentration of 3'-CTNU on the rate of inactivation is presented in Fig. 1B and no saturation kinetics were formed even when the amount present was 24 mM 3'-CTNU. Technical problems prevented inactivation studies at higher concentrations of 3'-CTNU.

The effect of 3'-CTNU, allosteric effectors and ribonucleotides on thymidine kinase activity. Table II shows that the allosteric effectors (dTTP, dCDP and

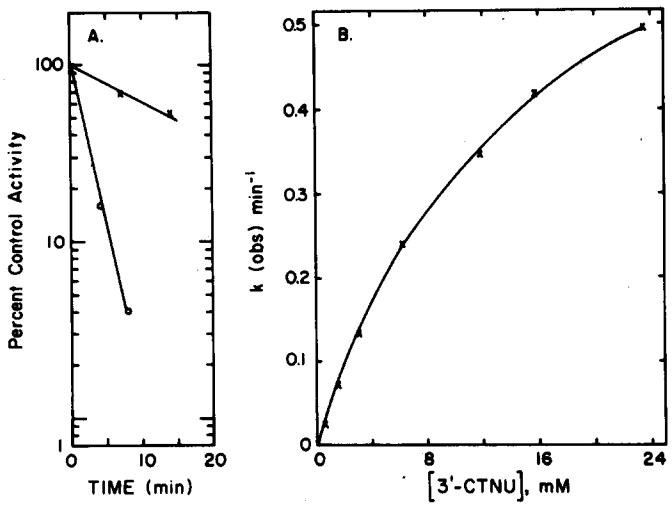

Fig. 1. A. Effect of 3'-CTNU on the activity of *E. coli* thymidine kinase. 3'-CTNU was added to an incubation mixture, composed of enzyme (0.18 units), 100 mM Tris-HCl, pH 7.5, and 2.5 mg bovine serum albumin/ml. At various times of incubation the remaining enzyme activity was assayed by the procedure described in the text, 0.78 mM 3'-CTNU (X———X); 15.7 mM 3'-CTNU (○———○). B. Effect of 3'-CTNU on the rate of inactivation of *E. coli* thymidine kinase. Incubation time was 20 min. The procedures used were the same as in Fig. 1A.

TABLE II

EFFECT OF VARIOUS ALLOSTERIC EFFECTORS AND RIBONUCLEOTIDES ON THE INACTIVATION OF *E. COLI* THYMIDINE KINASE BY 3'-CTNU

E. coli thymidine kinase was incubated at 37°C for 5 min with the indicated compounds shown in the table. 3'-CTNU was added to the incubation mixture which contained the enzyme (0.04 units), 100 mM Tris-HCl, pH 7.5, and 2.5 mg/ml bovine serum albumin. Thymidine kinase activity was assayed as described in the text. dCDP (6 mM) was added when the reaction mixture which contained dTTP was assayed for remaining thymidine kinase activity.

Concentration (mM)		Percent activity remaining
3'-CTNU	Nucleotide	
3.5		50
3.5	9 mM dCDP	99
3.5	9 mM dCTP	100
3.5	9 mM dTTP	106
3.5	9 mM CDP	64
3.5	9 mM CTP	68

TABLE III

EFFECT OF ATP · Mg^{2+} AND THYMIDINE ON THE INACTIVATION OF YEAST ALCOHOL DEHYDROGENASE BY 3'-CTNU

Yeast alcohol dehydrogenase (0.83 units) was incubated for 20 min at 22°C with the indicated compounds shown in the table. 3'-CTNU was added to the incubation mixture which contained enzyme (0.83 units), 0.01 M phosphate, pH 7.3, and 2.5 mg/ml bovine serum albumin. Alcohol dehydrogenase activity was assayed as described in the text.

Concentration (mM)			Percent activity remaining
3'-CTNU	ATP · Mg^{2+}	Thymidine	
7.8	0	0	42
7.8	9	0	42
7.8	0	0.75	35
7.8	9	0.75	43
0	0	0	99

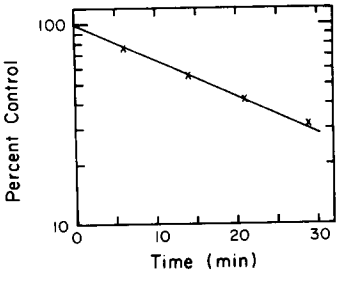

Fig. 2. Effect of 3'-CTNU on the rate of inactivation of yeast alcohol dehydrogenase. The enzyme (0.83 units) was incubated in 20 mM phosphate, pH 7.3, with 7.8 mM 3'-CTNU (X———X) at ambient temperature for various periods of time. The control reaction mixture contained 2.5 mg/ml bovine serum albumin, but no 3'CTNU. The remaining enzyme activity was assayed by the procedure described in the text.

dCTP) completely protected the enzyme from inactivation by 3′-CTNU, while the corresponding ribonucleotides (CDP and CTP) afforded only partial protection.

The effect of 3′-CTNU, ATP · Mg^{2+} and thymidine on alcohol dehydrogenase activity. The effect of 3′-CTNU, ATP · Mg^{2+} and thymidine on the activity of alcohol dehydrogenase is shown in Table III. ATP · Mg^{2+} alone or combined with thymidine, or thymidine alone did not alter the rate of enzyme inactivation by 3′-CTNU. The kinetics of alochol dehydrogenase inactivation activity by 3′-CTNU appears to follow first-order kinetics (Fig. 2).

Discussion

The first-order inactivation kinetics of *E. coli* thymidine kinase with 3′-CTNU indicate that the process of inactivation involves the formation of an inactive enzyme and not a less-active one. Since 3′-CTNU is a thymidine analog, it was unexpected to find that thymidine did not protect the enzyme from inactivation by the thymidine nitrosourea analog. This could be due to either 3′-CTNU or the inactivating product derived from 3′-CTNU having a very high affinity to the active site, since the concentration of thymidine used was more than 140-fold of the K_m value. However, the site of the inactivation may be other than the active site of the enzyme.

E. coli thymidine kinase was shown by Okazaki and Kornberg [9] to be a regulatory enzyme, the activity of which is affected by various nucleoside di- and triphosphates. They also showed that two molecules of ATP can bind to the enzyme molecule, one bound to the active site where it functions as a phosphate donor, and the other bound to the regulatory site as an allosteric activator. The regulation of enzyme activity by these allosteric regulators involves the dimerization of enzyme molecules by these effectors [10]. The activating allosteric regulators, dCTP and dCDP, increase the sedimentation coefficient of the enzyme from 3.5 S to 5.3 S and 5.5 S, respectively; while the inhibiting allosteric regulator, dTTP, increased the sedimentation coefficient of the enzyme to 6.0 S. In contrast, CDP and CTP increased the sedimentation coefficient of the enzyme from 3.5 S and 3.9 S and 4.2 S, respectively, which presumably indicates non-dimerization.

When the enzyme molecules are in the dimer form, whether induced by the activating or inhibiting regulatory effectors, they are completely resistant to the inactivating effect of 3′-CTNU. Thymidine per se does not induce dimerization of *E. coli* thymidine kinase. The partial protection afforded by CDP and CTP against 3′-CTNU could be due to a slight conformational change of the enzyme produced by these compounds which is manifest by the slight increase in sedimentation coefficient [10]. When the enzyme is in the dimer form the critical site of interaction of 3′-CTNU with the enzyme is not available. The regulatory site, if involved would be protected by the nucleotides already bound, and if another site is primarily affected it may not be exposed to the nitrosourea analog because of the marked change in conformation produced by the dimerization of *E. coli* thymidine kinase. Yeast alcohol dehydrogenase was used as a control to determine whether the protecting nucleotide might be functioning as a scavenger for reactive alkylating and carbamoylating products

of 3′-CTNU. However, this possibility is not probable, since ATP · Mg^{2+} alone or combined with thymidine, did not afford any protection to this enzyme against the inactivation by 3′-CTNU.

Previous studies by Cysyk and Prusoff [11] found the regulators dCDP (an activator) and dTTP (an inhibitor) significantly reduced the rate of inactivation of *E. coli* thymidine kinase to ultraviolet irradiations, but had no effect on the ultraviolet sensitivity of lysozyme irradiated under the same conditions. This protective effect was correlated to the ability of these compounds to produce dimerization of *E. coli* thymidine kinase. The allosteric regulators, dCDP and dTTP, also decreased the rate of photolytic inactivation of this enzyme when irradiated in the presence of various halogenated substrate analogs [11—14].

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